Disinhibiting neurons in the dorsomedial hypothalamus delays the onset of exertional fatigue and exhaustion in rats exercising in a warm environment

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Abstract

Stimulants cause hyperthermia, in part, by increasing heat generation through exercise. Stimulants also delay the onset of fatigue and exhaustion allowing animals to exercise longer. If used in a warm environment, this combination (increased exercise and decreased fatigue) can cause heat stroke. The dorsomedial hypothalamus (DMH) is involved in mediating locomotion from stimulants. Furthermore, inhibiting the DMH decreases locomotion and prevents hyperthermia in rats given stimulants in a warm environment. Whether the DMH is involved in mediating exercise-induced fatigue and exhaustion is not known. We hypothesized that disinhibiting neurons in the dorsomedial hypothalamus (DMH) would delay the onset of fatigue and exhaustion in animals exercising in a warm environment. To test this hypothesis, we used automated video tracking software to measure fatigue and exhaustion. In rats, using wearable mini-pumps, we demonstrated that disinhibiting the DMH, via bicuculline perfusion (5 µM), increased the duration of exercise in a warm environment as compared to control animals (25+/-3 min vs 15 +/-2 min). Bicuculline-perfused animals also had higher temperatures at exhaustion (41.4+/-0.2 ^{o}C vs 40.0+/-0.4 ^{o}C). Disinhibiting neurons in the DMH also increased the time to fatigue. Our data show that the same region of the hypothalamus that is involved in mediating locomotion to stimulants, is also involved in controlling exhaustion and fatigue. These findings have implications for understanding the cause and treatment of stimulant-induced-hyperthermia.

Keywords: exercise, exhaustion, dorsomedial hypothalamus

1. Introduction

Stimulant-induced-hyperthermia (SIH) is a devastating condition that can occur in persons using drugs such as methamphetamine (Kojima et al., 1984), MDMA (Henry et al., 1992; Liechti et al., 2005; Milroy et al., 1996), and cocaine (Menaker et al., 2011). As peak core temperatures increase, so does mortality (Gowing et al., 2002). Patients who develop SIH are often exerting themselves in warm environments like nightclubs or concert venues (Michael White, 2014; Ridpath et al., 2014). In addition, stimulants are used by athletes seeking a competitive edge (Reardon and Creado, 2014). Use of stimulants as ergogenic agents also increases the risk of persons developing exertional heat stroke (Bailes et al., 2002; Eichner, 2008; Eliason et al., 2012).

Motor activity is an important contributor to the development of SIH. As we showed, motor activity accounts for half of the rate of heat accumulation from the amphetamine MDMA (Zaretsky et al., 2014a). Stimulants also mask the onset of exertional fatigue (Zaretsky et al., 2014a). Fatigue serves as an important compensatory mechanism to prevent overexertion (Fuller et al., 1998; Noakes, 2012). The combination of increased motor activity and decreased fatigue increases the risk of developing exertional heat stroke.

The central mechanisms by which stimulants cause locomotion and prevent fatigue is not completely known. We have shown that inhibiting neurons in the region of the dorsomedial hypothalamus (DMH) decreases SIH and prevents mortality from MDMA given in a warm environment (Zaretsky et al., 2014b). This protection does not involve altering increases in non-shivering thermogenesis or decreases tail blood flow produced by MDMA (Zaretsky et al., 2014b). Rather, it involves decreasing MDMA-mediated locomotion (Zaretsky et al., 2014b; Zaretsky et al., 2015).

There is experimental evidence to suggest the DMH is involved in controlling fatigue and exhaustion. In animal models of inflammation and cancer, the neuropeptide orexin has been shown to be involved in the development of fatigue (Gaykema and Goehler, 2009; Grossberg et al., 2011; Weymann et al., 2014). The neurons that secrete this orexin are located, in part, in the DMH. These neurons are likewise activated by stimulants (Estabrooke et al., 2001; Fadel et al.,

2002). While there have been no studies specifically exploring the role of the DMH in exhaustion, there have been studies looking at the ventromedial hypothalamus (VMH), a brain region just ventral to the DMH. In rat models, inhibition of neuronal activity in the VMH decreases the time to fatigue and inhibits the metabolic support of exercise (Guimaraes et al., 2013; Miyaki et al., 2011; Wanner et al., 2011). Based on its proximity to the DMH, and the microinjection volumes and concentrations involved in these experiments, it is possible that findings attributed to the VMH could also involve neurons in the DMH. Furthermore, neurons in the DMH are known to project to the VMH and vice versa (Ter Horst and Luiten, 1987).

We hypothesized that disinhibiting neurons in the region of the DMH would increase the time to fatigue and exhaustion in rats exercising in a warm environment. To study this, we created an innovative method using video tracking software to objectively measure the time to fatigue and exhaustion. In addition, we modulated neuronal activity in the DMH by reverse dialyzing the $GABA_A$ antagonist bicuculline (BMI) (Horiuchi et al., 2009; Hunt et al., 2010; Zaretsky et al., 2014b) in exercising rats wearing programmable mini-pumps. The results of these experiments are important in furthering our understanding of the central mechanisms behind SIH.

2. Results

2.1. Dose response of BMI perfused through the DMH

The baseline parameters were within the range of those typical seen for conscious Sprague-Dawley rats measured during the day (Zaretsky et al., 2011). Perfusion of the DMH with BMI evoked responses similar to those seen after microinjection of BMI (Zaretskaia et al., 2002): locomotor activation, tachycardia, moderate hypertension and hyperthermia (Fig.1). These changes were observed within the first minute of perfusion with the highest studied dose (20 µM). The responses were clearly dependent on the concentration of BMI: The lowest dose (2 µM) evoked barely detectable responses, while perfusion of 20 µM BMI resulted in a heart rates in the range of 480-500 beats/min (Fig.1). No abnormal behaviors were noted in any of the animals. When the pump was deactivated, locomotion, heart rate, and blood pressure returned to baseline after approximately 20 min (Fig.1). Core body temperature demonstrated significant temporal inertia, but eventually returned to baseline. For the subsequent treadmill experiments we used the 5µM dose as it clearly produced responses, but not so extreme as to likely decrease the ability of rats to run effectively.

2.2. Activation of neurons in the DMH increases the time to fatigue and exhaustion, and the temperature at fatigue and exhaustion

The initial core body temperature of rats, before any manipulations occurred, was not different between animals perfused with artificial CSF (aCSF) and BMI (Fig.2A, B). The effect of BMI on body temperature was different from aCSF over time (Fig.2B, $F(3,36)=4.1$, $p<0.05$). Insertion of the microdialysis probe (noted by the line labeled perfusion in Fig.2A, which required handling of the rat outside of the cage, resulted in a slight increase in body temperature. Perfusion of BMI for 30 min (noted by the line treadmill in Fig.2A) resulted in a statistically significant increase in core body temperature (Fig.2B, 37.4 ± 0.2^{o}C at baseline vs 38.1 ± 0.2^{o}C before exercise, $p<0.05$). In rats perfused with aCSF, core body temperatures immediately prior to the onset of treadmill running were not different from their baseline (37.3 ± 0.1^{o}C at baseline vs 37.6 ± 0.1^{o}C before exercise, $p>0.1$). Running in a warm environment resulted in a rapid rise in core body temperature (Fig.2A). During the initial 7-8 min, rats in both experimental groups were able to maintain a running position at the front of the treadmill (Fig.2C). BMI statistically significantly affected times to fatigue and exhaustion (Fig.2D; $F(1,12)= 8.3$; $p<0.05$). Compared to aCSF

animals, animals perfused with BMI, ran 5.9 minutes longer until fatigue occurred (Fig.2D, $p<0.05$). The increased time to fatigue in the BMI perfused animals was accompanied by corresponding increases in core body temperatures at fatigue (Fig. 2B; 40.3±0.3^{o}C vs 39.1±0.2^{o}C; $p<0.01$). Compared to the aCSF animals, the time at which exhaustion occurred was increased by 9 minutes in the BMI-perfused animals (Fig. 2D; 15.2±1.8 min vs 24.6±3.1min; $p<0.05$). This time increase occurred even though the perfusion with BMI significantly increased body temperature prior to the onset of treadmill running. The mean core body temperature at exhaustion of BMI-perfused rats was also significantly higher than aCSF control animals: 41.4±0.2^{o}C compared to 40.0±0.4^{o}C (Fig.2B; $p<0.05$).

3. DISCUSSION

This study confirmed our core hypothesis that disinhibition of neuronal activity in the region of the dorsomedial hypothalamus increases the time to fatigue and exhaustion in rats exercising in a warm environment. An important finding in our study, that has clinical implications, is that delaying the onset of fatigue and exhaustion in a warm environment, comes at the expense of core body temperature. In our experiments BMI perfused animals exercised longer. This occurred even though, compared aCSF animals, they began exercise with higher body temperatures– something that should have decreased their exercise times (Gonzalez-Alonso et al., 1999; Walters et al., 2000; Zaretsky et al., 2015). It is not known if the effects on exercise seen from BMI perfusion are specific to a warm environment. Amphetamines have been shown to increase time to exhaustion in rats at both warm and room temperature (Gerald, 1978; Zaretsky et al., 2014a). Future research is needed to determine if hypothalamic disinhibition would likewise increase running times at warm and lower ambient temperatures (e.g., room temperature or in the cold). Our findings that hypothalamic disinhibition causes animals to run longer in the warm, and that this increased time of exertion is associated with significant increases in core body temperature, is similar to what has been shown in humans given stimulants while exercising in a warm environment (Roelands et al., 2008; Watson et al., 2005). Most concerning from our study is the finding that the core body temperature at exhaustion is close to values that are able to damage thermosensitive tissues (Kregel, 2002). Together these data suggest that stimulants activate the DMH thereby delaying the onset of fatigue and exhaustion and, when used in warm environment, can increase the risk of exertional heat stroke. This may have implications to a variety of clinically relevant situations.

Persons can develop SIH in several different contexts. Hyperthermia from the drug MDMA often occurs in persons who have used it while dancing at parties or outdoor concerts (Henry, 1992) - venues that are often hot and crowded. Deaths from cocaine use also occur more commonly on warm days (>24˚C) (Bohnert et al., 2010; Marzuk et al., 1998). Additionally, deaths occurring in police custody happen more often on warm days in persons who use stimulants and aggressively resist arrest (Ross, 1998; Ruttenber et al., 1997; Stratton et al., 2001). Stimulants are also used as ergogenic aids. When used on warm days, they also increase the risk of exertional heat stroke (Bailes et al., 2002; Eichner, 2008; Eliason et al., 2012)

(Eichner, 2008). The finding that a key contributor to the development of SIH may be the failure to develop fatigue and exhaustion, offers new approaches to prevention and treatment. Since persons using stimulants may not be able to recognize when they are overheating (Crandall et al., 2002), prevention of SIH may require thermal imaging to identify persons with critically elevated body temperatures. Although not widely used, this technology has been shown to be useful in controlled settings of exertion (Bourlai et al., 2012). In the future, technology like this could be employed at sports events, concerts, and by first responders to easily assess body temperature in persons at risk of SIH.

Identifying the brain regions involved in mediating SIH may also offer new treatment approaches. Currently patients with SIH are treated by giving them high concentrations of sedatives such as benzodiazepines. These drugs, however, require repeat dosing, and take a significant amount of time to achieve adequate sedation (Nobay et al., 2004; Takeuchi et al., 2011). By identifying what neurons and neurotransmitters are affected by stimulants, and how this is in turn is responsible for increasing motor activity and preventing fatigue and exhaustion, may lead to new more specific treatments. The DMH has a mixed population of GABAergic and Glutamatergic neurons (Myers et al., 2014). These neurons project to a variety of brain regions involved in stress response, cardiovascular regulation, and thermoregulation (Myers et al., 2014). As the technique used in our study, reverse dialysis, would activate both groups of neurons, it is not possible to know if the effects we show are from increasing glutamatergic tone or increasing GABAergic tone. Another potential target to explore in DMH are dopamine neurons. The DMH region contains tyrosine hydroxylase (TH) positive neurons (Barraud et al., 2010; Bjorklund and Skagerberg, 1979; Lindvall et al., 1983). Anatomically this group, called A11 neurons, are dispersed above the third ventricle in the region of the DMH and send projections to the prefrontal cortex (Takada et al., 1988), the dorsal raphe nucleus (Peyron et al., 1995). They are the sole source of dopamine input to the spinal cord (Barraud et al., 2010; Bjorklund and Skagerberg, 1979; Lindvall et al., 1983; Qu et al., 2006). These neurons may be of particular relevance to fatigue and exhaustion. Studies in humans and rodents has shown that bupropion and methylphenidate, which increase central concentrations of dopamine and norepinephrine, delay the onset of fatigue, and increases the temperature at fatigue in humans and rodents exercising in a warm environment (Hasegawa et al., 2008; Roelands et al., 2008; Watson et al., 2005). Norepinephrine receptors are another potential target to explore. Within the

hypothalamus, the DMH has one of the highest concentrations of NE (Bernardis and Bellinger, 1987) and it receives neural afferent impulses from many brain stem NE groups, including the locus coeruleus (Moore and Bloom, 1978). The DMH also contains large concentration of alpha-1 receptors (Strazielle et al., 1999). Previous research has also shown that microinjections of bicuculline into the DMH increases extracellular concentrations of NE (Shekhar 2002). Another group of neurons with a link to exhaustion are the orexin-containing neurons. Located exclusively in the lateral hypothalamus and DMH (Peyron et al., 1998), they are excited by amphetamines and stimulants (Estabrooke et al., 2001; Fadel et al., 2002; Scammell et al., 2000), and are involved in mediating exhaustion caused by infection and cancer (Gaykema and Goehler, 2009; Grossberg et al., 2011). We have previously shown that the excitatory effects caused by amphetamines are mediated, in part, by the orexinergic system (Behrouzvaziri et al., 2015; Rusyniak et al., 2012). All of the potential targets discussed above are worthy of investigation.

Although we believe the effects that BMI has on exercise are mediated by neurons located predominantly in the DMH, we acknowledge that these studies do not definitely rule out a role of neurons in adjacent brain regions like the VMH. As seen in Fig 5, the tips of the microdialysis cannulas in several animals ended in the dorsal aspect of the VMH. Although the tip of the microdialysis cannula is solid and therefore does not exchange dialysate, it is possible that some BMI may have either been released in part of the VMH or may have diffused over time into the region of the VMH. Based on the location of the cannulas, the vast majority of the microdialysis membrane is located within the region of the DMH. Future studies are needed to confirm the precise location and identity of the neurons mediating fatigue and exhaustion. Our work provides an important anatomic starting point for these studies.

In conclusion, we demonstrated that activation of neurons in the DMH delays the onset of fatigue and exhaustion in animals running in a warm environment. This comes at the expense, of core body temperature. Stimulants, like amphetamines, which likewise activate neurons in the DMH, may increase the risk of developing exertional heat stroke in persons exerting themselves in a warm environment. Determining how stimulants act in the DMH, holds the potential to develop new prevention strategies and treatments for SIH.

4. Experimental Procedures

4.1. Animals

Male adult Sprague-Dawley rats (weight 300 ± 20 g; Harlan, Indianapolis, IN) were used in this study. All procedures were approved by the Indiana University Animal Care and Use Committee. Experiments were performed using single-housed rats that were maintained in a 12 h light/dark cycle (7a to 7p) and fed ad libitum. We conducted experiments on fully conscious rats between 10:00 a.m. and 4:00 p.m. Experiments were performed in a custom-made environmental chamber, which housed a treadmill and telemetric receivers for recording physiological parameters of a rat in a home cage or on a treadmill.

4.2. Surgical procedures

4.2.1. Anesthesia

Animals were anesthetized with 1.5-2% isoflurane in oxygen, with isoflurane concentration adjusted as needed. Heart rate and oxygen saturation were monitored during surgery using Pulse Oximeter monitor (LS1P-10R, Nonin, Plymouth, MN).

4.2.2. Guide cannulas implantation

Animals were placed in a stereotaxic apparatus with the incisor bar set at 3.3 mm below the interaural line. The skin overlying the dorsal surface of the skull was pretreated with lidocaine/epinephrine mixture, cut, and retracted followed by removal of soft tissue to expose the surface of the skull. The skull was treated with a 30% hydrogen peroxide solution using cotton-tipped applicators. This stopped bleeding, aided in sterility, and enhanced the visibility of sutures used as stereotaxic landmarks. Using a rotary tool (MiniMite Cordless 4.8V, Dremel, Racine, WI) equipped with a surgical carbide burr (DHP557, Miltex, Plainsboro, NJ), we made a small hole in the skull. We inserted the guide cannula (to fit CMA11 microdialysis probe, Harvard Apparatus, MA) through the hole, and positioned it to target the left DMH, so that the tip of the probe, considering 2 mm working length of the probe, was positioned: AP -3.1 mm; LR -0.5 mm; HD -9.0 mm using bregma as a reference point (Paxinos and Watson, 1998). The left DMH was chosen as microinjections of bicuculline into this side of the DMH elicit greater locomotor responses, than the right, (Zaretskaia et al., 2002). We used a vertical approach with special care

to avoid the central sinus and placed two jeweler's screws (size 80) into the skull to facilitate attachment of the cement cap. Guide cannulas were secured using Vetbond glue (3M, St. Paul, MN) and cranioplastic cement. A dummy cannula (CMA Microdialysis AB, Sweden) was inserted into the guide during the surgery and was left in place until the experiment.

4.2.3. Implantation of telemetric probes to measure core body temperature

For treadmill experiments a telemetric probe (TA-F40 telemetric transmitters, Data Sciences Int, St. Paul, MN) was placed intraperitoneally as previously described (Zaretsky et al., 2014b), and rats were returned to their home cage for at least 7 days to recover.

4.2.4. Implantation of telemetric probes to measure core body temperature, heart rate and blood pressure

For pilot work to determine the dose of BMI to be used in treadmill studies, a telemetric probe (C50-PXT, Data Sciences Int., St. Paul, MN) for the measurement of heart rate, blood pressure, core body temperature and locomotor activity was implanted into the abdominal cavity according to manufacturer's recommendations as we have previously described (Zaretsky et al., 2011). The tip of catheter was inserted into abdominal aorta through the femoral artery.

4.3. **Intracranial perfusion**

To perfuse the microdialysis probe we used implantable programmable minipumps (iPrecio SMP-200, Primetech Corp., Japan). The SEBS tubing outlet of the pump was shortened to 1 cm, and 7 cm piece of FEP tubing (I.D.=0.12 mm, CMA Microdialysis AB, Sweden) was connected to it. The inlet of microdialysis probe (CMA 11) was connected to FEP tubing through 5 mm piece of microbore PVC tubing (I.D.=0.5 mm, Small Parts Inc., Miami Lakes, FL). A short piece (5-10 mm) of microbore PVC tubing was connected to the outlet of the microdialysis probe to prevent it from drying during any interruption of the flow. The body of the pump was placed into the container that can be attached to a modified infusion saddle (Kent Scientific Corp, Torrington, CT). The container was made from two pieces of transparent plastic, which when placed together, formed a rectangular cuboid (38x19x9 mm) that fit snugly around the body of iPrecio minipump. When the pump is placed inside the container, two plastic components of the container were fixed together with narrow adhesive tape, while the outlet tubing extended

outside the container through the slit. The "bottom" part of the container had a registered jack RJ11 attached. The 6P2C modular plug matching the RJ11 jack was attached to the infusion saddle. Latching the tab allowed connecting and disconnecting the container to and from the saddle in one click. Both the RJ11 and the modular plug were sawed from unnecessary components to decrease the weight and the total height of the assembly when positioned on the back of a rat (Fig.3A and 3B).

The pump reservoir was filled with either aCSF or BMI, depending on group allocation, and the pump was programmed to deliver a continuous flow at 30 µl/h. To prime the system the pump was attached to a microdialysis probe outside of the animal and perfused for at least 2 h in a beaker with sterile saline. The flow was then stopped, and the pump, which was still connected to the microdialysis probe, was programmed to follow the experimental protocol. The body of the pump was placed into the container. Using gentle restraint of the rats, the dummy cannula was removed from guide. The microdialysis probe was inserted into the guide cannula, and the container with the pump was attached to the saddle. The animal was returned to the home cage, and the experiment proceeded.

4.4. **Treadmill protocol**

Prior to treadmill experiments, rats were familiarized to running on a motorized rodent treadmill (Columbus Instruments, Columbus, OH) set with 5% incline as we have previously described (Zaretsky et al., 2014a; Zaretsky et al., 2015). Briefly, on the first day of familiarization, the rats were placed on the treadmill with the belt speed set at standby and were given 10 min to explore the surroundings. After 10 min the treadmill speed was set to 6 m/min for 5 min. In next four days rats were subjected to five-minute sessions that included running at progressively increasing speeds to a maximum of 18 m/min by day 5. Previous studies have shown that the workloads of familiarization programs accustomed the rats to treadmill running but are not sufficient to induce training adaptations (Lambert and Noakes, 1989). Mild electric stimulus at the back of the treadmill chamber promoted learning of running behavior. Rats undergoing the familiarization procedure did so wearing the saddle. Rats were fitted with a saddle under light isoflurane anesthesia (1.5%) before familiarization to run on the treadmill.

4.5. **Measuring fatigue and exhaustion**

The subjectivity behind the terms fatigue and exhaustion has led many authors to use them interchangeably. For our experiments, we defined them separately. Exhaustion was defined as the point in which the animal is no longer able to run despite prodding. Fatigue was defined as the point in which the animals could not consistently keep up with the treadmill speed but was still able to run. This is similar to how others have defined it in experiments (Rodrigues et al., 2009; Soares et al., 2004) and is similar to what is reported in humans (Davis and Bailey, 1997). We measured fatigue and exhaustion by modifying a previously published method (Guasch et al., 2013). Instead of relying on an observer to intermittently score the animals running quality, we used a video-tracking system software (Anymaze, Stoelting Co., Wood Dale, IL). Video was recorded using a USB-camera connected to a laptop that ran Xtrasense software (surveillance computer-based system, XmediaStudio, Dawang group) using a proprietary codec. The timer of the computer was embedded into the video stream, which was set at the resolution 640x480. To improve make the contrast between the rat and the environment for the video tracking, we positioned the video camera from the side of the treadmill and covered the opposite face of the treadmill lane with dark paper. To measure running quality with the video-tracking software we used a built-in function to measure the distance of the animal's center from the back of the treadmill. One-minute averages of the normalized position on the treadmill were calculated by video tracking software.

Normally, at the start of exercise, rats run at the front of the treadmill. Over time the rat is unable to keep up with the treadmill (what we, and others term fatigue) (Rodrigues et al., 2009; Soares et al., 2004) and will slide toward the back. Before reaching the back, the rat will quickly run to the front the treadmill again. It will repeat this cycle until it nears exhaustion and is unable to get back to the front of the treadmill. This can be objectively recorded using video tracking software. The automatic tracking analysis of the video recording by Anymaze creates a continuous measure of rat position on the treadmill relative to the back of treadmill (Fig.4A and B, top graph). The length of treadmill belt is 0.4 m. However due to the length of rat itself, the range of possible positions is between 0.1 m and 0.33 m. These minimal and maximal values are estimated from the completed recording and were used to calculate the normalized position at the treadmill (with values between 0 and 1, Fig.4, bottom graph). Fatigue was defined as when the

one-minute average center of the animal fell below the 0.7 boundary (Fig. 4A and B, bottom graph). Exhaustion was defined as when it fell below 0.35 (Fig. 4A and B, bottom graph). In some individual rats, average values fell below 0.7 for a short period of time, and then scores quickly returned to above 0.7 for prolonged time. To avoid underestimation of time to fatigue, time to fatigue was selected when the average position for one-minute falls below 0.7 and did not recover for at least three minutes. Our criteria are similar to that used in manual scoring (Guasch et al., 2013). A similar rule for exhaustion was used. If, however, the time between when the animal fell below 0.35 and could no longer run was less than 3 min, which was the case in a majority of animals, the time when the animal fell below 0.35 was considered the time to exhaustion.

4.6. **Experimental protocols**

4.6.1. Dose response BMI perfusion

Previous work from our laboratory has shown that disinhibiting neurons in the DMH can cause significant increases in heart, blood pressure, temperature, and can cause behavioral changes – effects that could affect the ability of the animal to exercise. Therefore, we did preliminary work to determine a concentration of BMI that could be perfused that would activate locomotion without causing significant behavioral disturbances or extreme autonomic changes. Experiments were performed at room temperature (24±1^{o}C). On the day of the experiment, we randomly assigned rats (n=3) to be perfused with one of three concentrations of bicuculline (2, 5 or 20 µM solution). During these experiments, we recorded telemetric parameters. The microdialysis perfusion was performed as described above in section 4.3. The animal was monitored for 60 min. In addition, we continued the recording for 30 min after the perfusion stopped. At the end of experiment the animal was gently restrained, the pump was disconnected, and the microdialysis probe was removed. Animals were crossed-over for each dose of BMI with experiments performed on alternate days with random selection of the concentration for perfusion.

4.6.2. Ergogenic effects of BMI perfusion

On the day of the experiment, rats, (n=7 in both groups), were brought to the experimental room and placed inside the custom-made environmental chamber. They were allowed to acclimate to

the new environment for at least 60 min. The custom-made environmental chamber temperature was set to 32±1°C (humidity below 20%). The core body temperature of rats was continuously monitored using implanted telemetric probes. The telemetric system was configured to record from a distributed receiver array, which had one receiver for the home cage outside the environmental chamber; one receiver was placed inside the chamber (for the recording in the home cage); and, two receivers at the top of the treadmill lane. This configuration allowed uninterrupted recording of core body temperature during transfers of an animal between the home cage and treadmill.

The minipump was filled with either artificial cerebro-spinal fluid (aCSF) or a 5 µM solution of bicuculline dissolved in aCSF, and was programmed to continuously deliver a flow at 30 µl/h. The pump was allowed to perfuse the connected microdialysis probe in a beaker with sterile saline for at least 2 h until the start of the experiment. The rat, in its home cage, was placed on the receiver of the telemetric system at room temperature (24±1°C, humidity 30-70%) outside of the environmental chamber, and the recording was started. The rat was allowed to adapt to this new lab environment for at least 60 min. After this period of time, the perfusion system containing either aCSF or 5 µM BMI was connected to the rat. The animal was returned to its home cage and the cage and rat were placed inside of the custom-made environmental chamber at a temperature of 32±1°C (humidity below 20%). The chamber also housed the treadmill. Telemetric recordings were begun and after 30 min, the rat was transferred from its cage to the treadmill lane at a 5˚ incline. The belt speed was quickly increased to 18 m/min. The core body temperature of the rats was continuously monitored, and rats were allowed to run until they were unable to keep up with the treadmill and received three continuous electric shocks on the grid at the back of the treadmill (frequency was set at 3 Hz). The belt was then stopped, and the experiment concluded. The rat was taken immediately from the lane and was transferred to the home cage at room temperature. In experiments measuring time to exhaustion, animals were only tested once.

4.7. **Verification of injection sites**

After the experiments were finished, animals were sacrificed by an overdose of pentobarbital (100 mg/kg, i.p.). Brains were perfused transcardially with cold saline, followed by 4%

paraformaldehyde (PF). Brains were then removed, fixed overnight in 4% PF, followed by placing them in a saturated 23% sucrose in PBS solution, and frozen. Brain sections (40 µm) were prepared using a cryocut (Leica 1850). The microdialysis cannula sites were determined by a "blinded" observer who reviewed brain sections with the fluorescent stain DAPI (4',6-diamidino-2-phenylindole) using anatomical landmarks according to a rat brain atlas (Paxinos and Watson, 1998). The sites of the microdialysis cannula were considered located in the DMH if the active 2mm of the microdialysis membrane fell within the region of the DMH as we have previously defined (Fig.5C) (Rusyniak et al., 2007). Figure 5 shows the positions of the cannulas for both the pilot study (Fig.5A) and treadmill experiments (Fig.5B).

4.8. **Data analysis and statistical procedures**

Statistical analyses and graphing were performed using Prism (GraphPad Software, San Diego, CA) software. The comparisons between groups (time to exhaustion, time to fatigue, and temperature at fatigue/exhaustion) were performed using analysis of variance (ANOVA) with a least significant difference (LSD) post hoc analysis where indicated. Significance was defined as a p-value <0.05. Values are presented as means ±SEM.

4.9. **Chemicals**

Bicuculline methiodide was dissolved in aCSF and stored at -20˚C until the time of the experiment. Artificial CSF was made using 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl2, 1.26 mM CaCl2, 1.2 mM Na2HPO4, and 0.3 mM NaH2PO4, pH 7.4. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Acknowledgements

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work. All designated authors qualify for authorship and all who qualify for authorship are listed. Special thanks to Dr. William Truitt for use of his AnyMaze video tracking system.

Funding

Research reported in this publication was supported by the National Institute on Drug Abuse of the NIH under award number R01DA026867. Furthermore, this work was conducted in a facility constructed under support from the National Center for Research Resources, of the NIH under award number C06 RR015481-010.

Competing interests and conflicts of interest:

The authors have no competing interests or conflicts of interest

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Figure Legends

Figure 1. Effect of microdialysis perfusion in the DMH with solutions of BMI (2, 5, 20 μM) on locomotor activity, heart rate, mean blood pressure, and core body temperature of rats in home cages at ambient temperature. The pump was activated at t=0 min and stopped at t=18 min, interval of perfusion is marked with the grey bar. Perfusion rate was 30 μl/h. Animals (n=3) were crossed over into each group with a washout period between experiments. All data points represent means ±SEM.

Figure 2. Effect of perfusing the DMH with BMI (5 μM) on treadmill running. Microdialysis probes perfused continuously with aCSF (open circles or white columns) or BMI (closed circles or black columns). Probes were inserted at t=-30 min. At t=0 min rats (n=7 per group) were transferred to the lane of treadmill set at 5^{o} incline and the belt was activated to the speed of 18 m/min. Rats ran until exhaustion. Dynamic data (curves A and B) only show data at the time points where all members of the group were still exercising (i.e., no animal had reached exhaustion). **A:** Core body temperature; **B:** statistical comparison of the core body temperature at different time points; **C:** position on the treadmill over time; **D:** time to fatigue and exhaustion. All data points and bars represent means ±SEM.

***** - $p<0.05$ between aCSF and BMI;

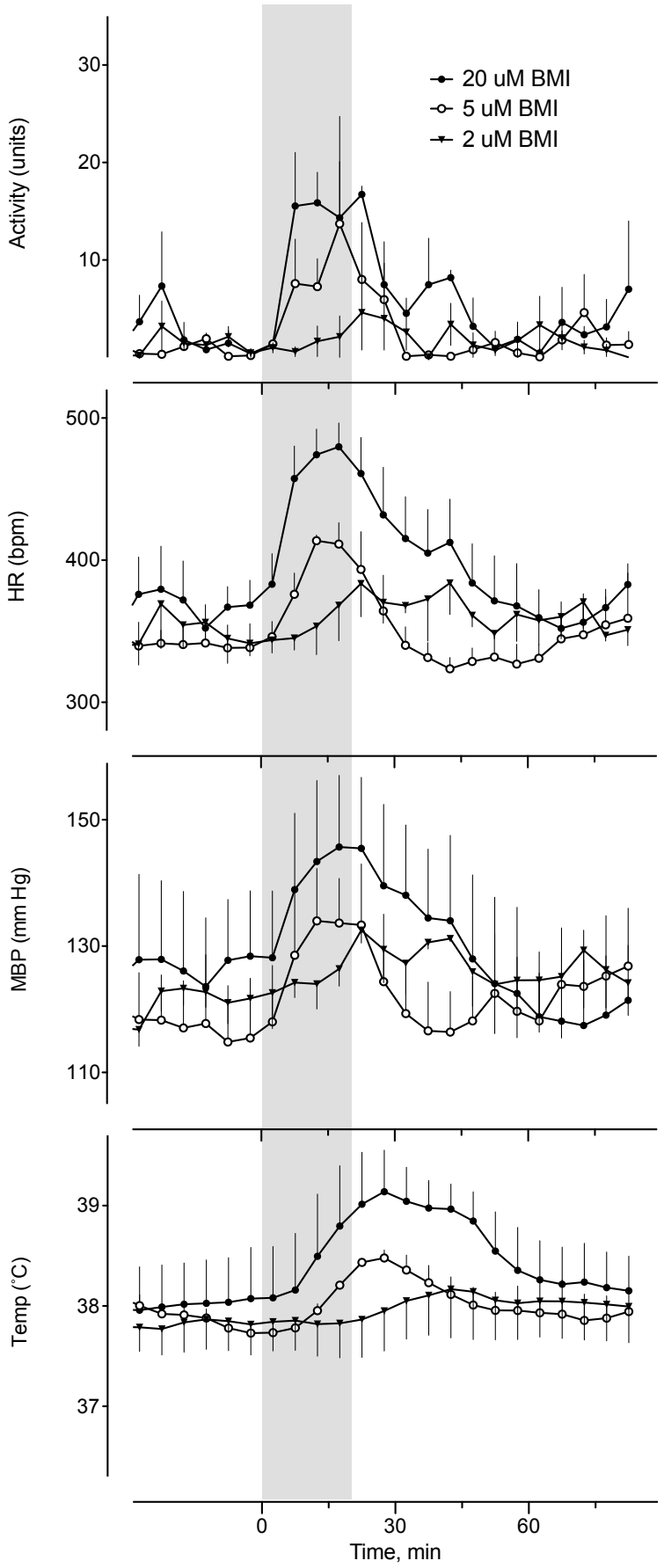
- $p<0.05$ compared with corresponding baseline

Figure 3. A: Schematic of rat running with infusion setup. **B:** Components of infusion setup. 1 – saddle (Kent Scientific, Torrington, CT), 2 – plastic container for a minipump, which consisted of two parts taped together, 3 – RJ-11 connector fitting 6P2C modular plug (4), 5 – microdialysis probe, 6 – low internal volume Teflon tubing. Art credit: Maria V.Zaretskaia, M.D.

Figure 4. A representative example of automatic tracking of individual rats running on the treadmill from the aCSF and BMI groups. Top – raw data; bottom – one-minute means for normalized data. In Fig.4A notice that although the mean position on animal on the treadmill

dropped below the predetermined cut off for fatigue (<0.7) the animal recovered before 3 minutes with average position above 0.7 (Fig.4A, bottom graph). A similar example (grey square) is presented for exhaustion (Fig. 4B, bottom graph).In both A and B the time of fatigue is denoted by a black square and the time of exhaustion as a grey square.

Figure 5. The placement of microdialysis probes. **A:** Placement of probes in preliminary work to determine the dose of BMI; **B:** Placement of probes for experiments testing the effect of BMI on exertional fatigue and exhaustion in a warm environment; **C:** Representative example of a brain section from an individual rate. We considered the placement successful if active zone (2mm membrane) fell within the DMH. f – fornix, mt – mamillothalamic tract, VMH – ventromedial hypothalamic nucleus, DMH – dorsomedial hypothalamus, DMC – dorsomedial hypothalamus, zona compacta.


Activity (units)
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2 uM BMI
HR (bpm)
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MBP (mm Hg)
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110
Temp (°C)
39
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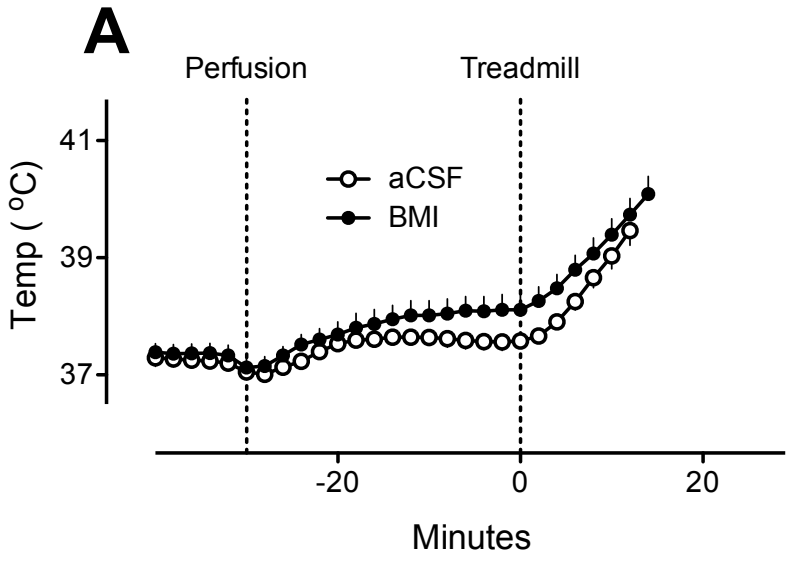
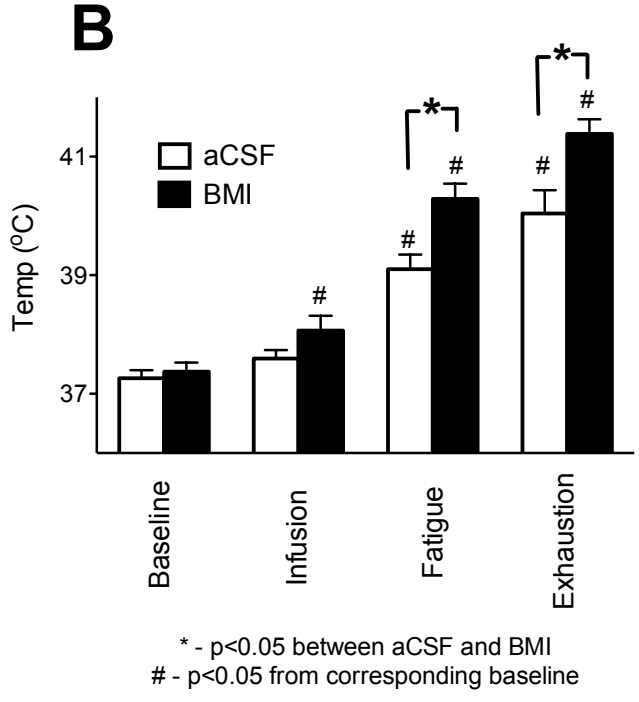
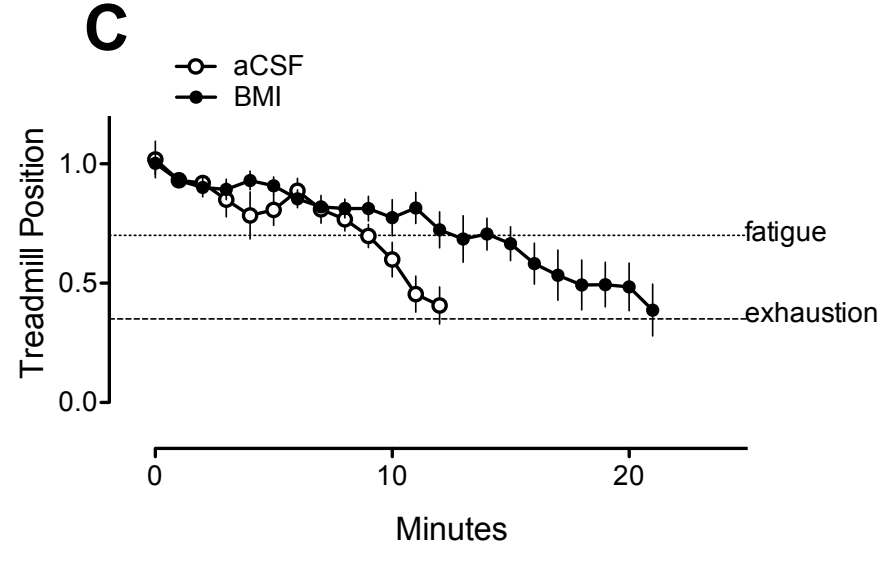
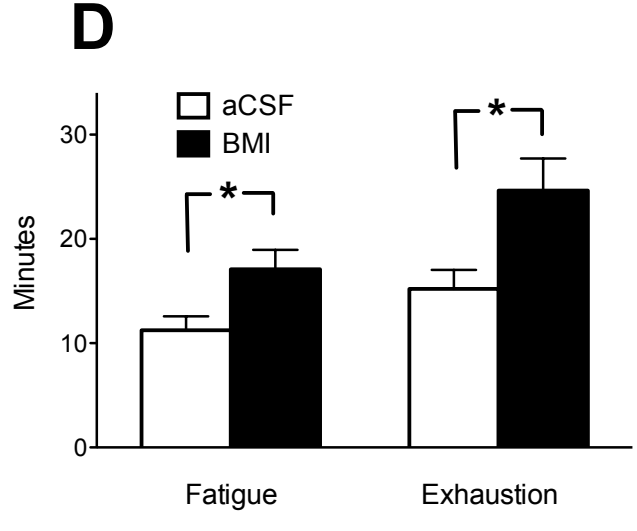
30
60
Time, min



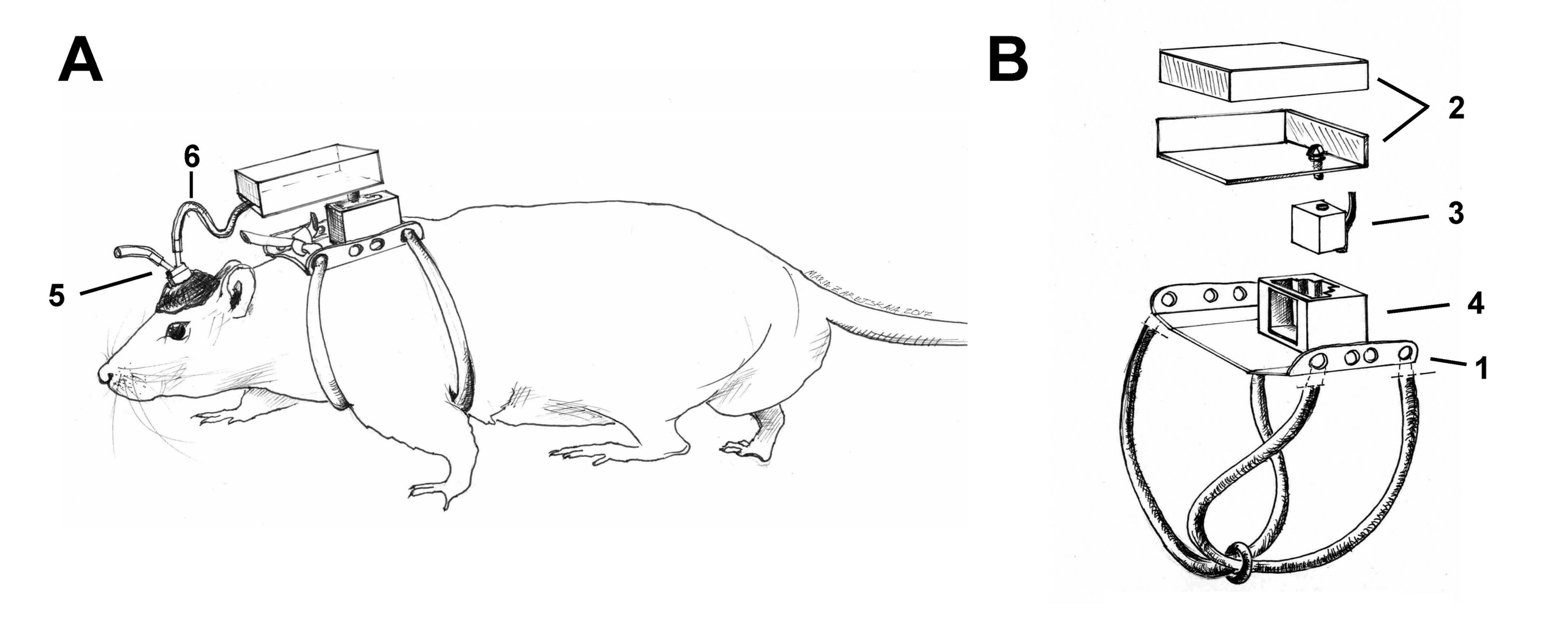


* - p<0.05 between aCSF and BMI
# - p<0.05 from corresponding baseline





* - p<0.05 between aCSF and BMI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MARIO ZARETSKAIA 2017
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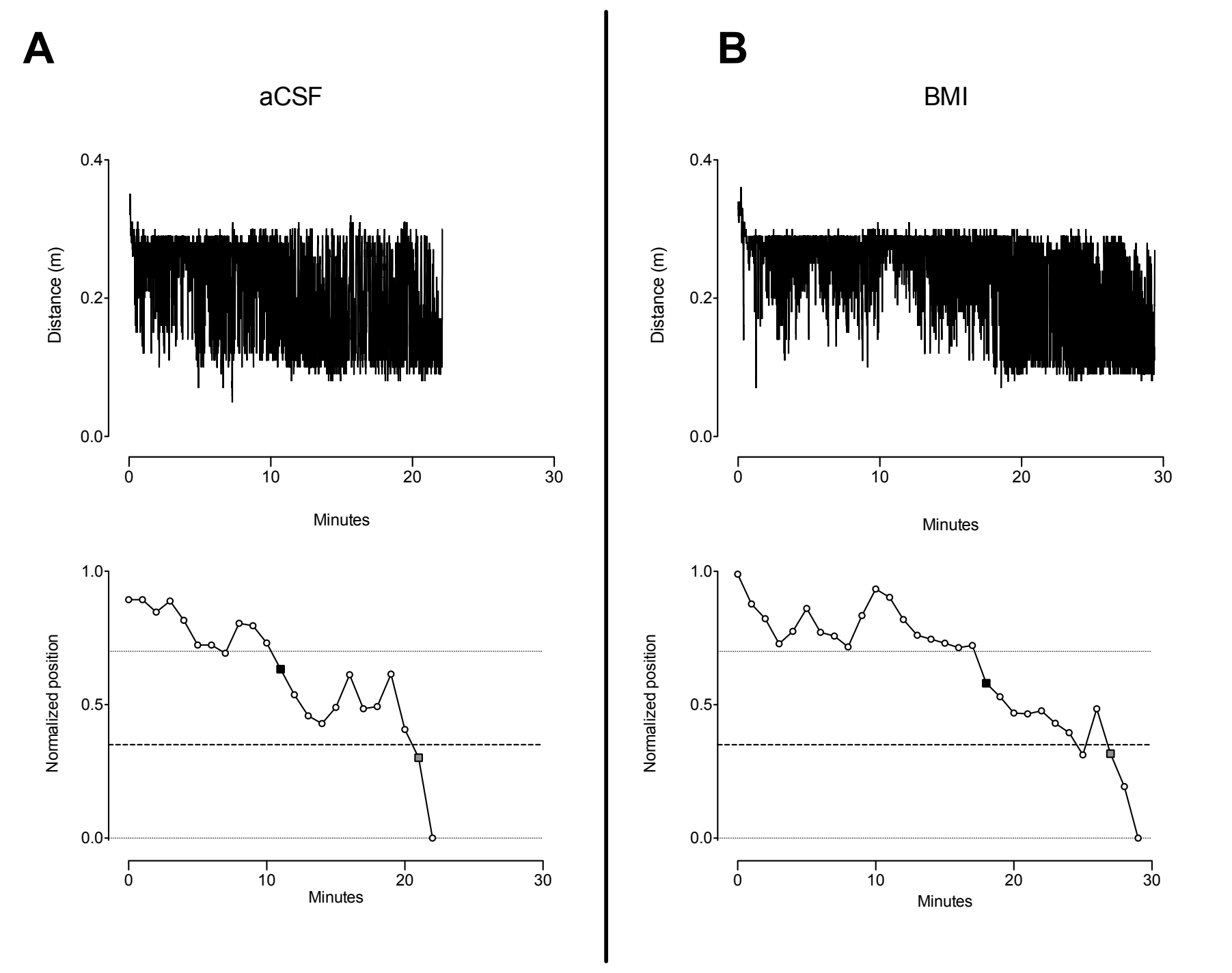
A
aCSF
Distance (m)
0.4
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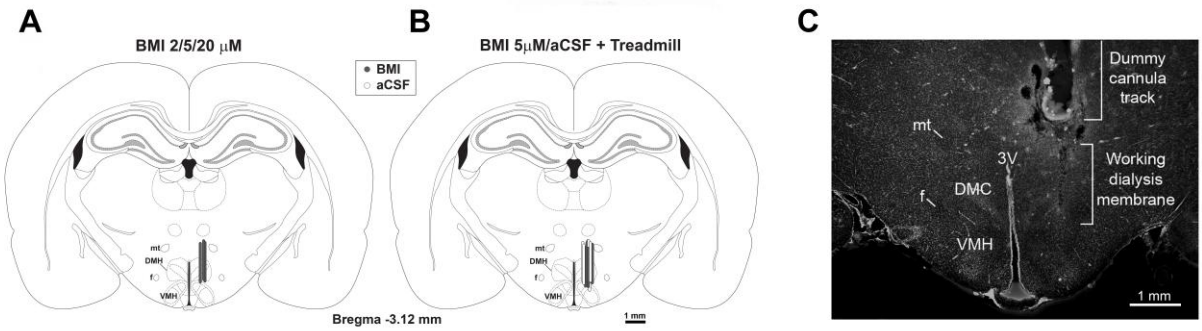

Highlights

- Hypothalamic neurons control warm exercise fatigue and exhaustion
- Delaying exhaustion in a warm environment increases body temperature at exhaustion
- Fatigue and exhaustion can be objectively measured using video tracking software